FINAL REPORT

U.S. Department of Energy

HUMAN GENETIC MARKER FOR RESISTANCE TO RADIATIONS AND CHEMICALS

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Institution: Columbia University

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Grant Project Officers: Dr. T. Wade Hillebrant

Project Duration: 09/15/96-09/14/00

2. **Table of Contents**

3. **Executive Summary:**

The purpose of this project has been to characterize the human *HRAD9* gene and evaluate its potential as a biomarker to predict susceptibility to the deleterious health effects potentially caused by exposure to radiations or chemicals present at DOE hazardous waste cleanup sites. *HRAD9* is a human gene that is highly conserved throughout evolution. Related genes have been isolated from yeasts and mice, underscoring its biological significance. Most of our previous work involved characteriztion of the yeast gene cognate, wherein it was determined that the corresponding protein plays a significant role in promoting resistance of cells to radiations and chemicals, and in particular, controlling cell growth in response to DNA damage. The latter process is called checkpoint control, and involves a transient delay in cell growth in response to DNA damage. It is believed that this delay allows extra time for cells to repair DNA damage before entry into crtitical phases of the cell cycle, such as mitosis. This project had two major parts. The first concerned the molecular characterization of the gene, including DNA sequence analyses and expression in cells and tissues. The second part involved a more functional characteracterization and focused on the role of *HRAD9* in radio/chemoresponsiveness and cancer. During the last year of the grant, we learned a lot about the function of *HRAD9*. We learned that the C-terminal region of the encoded protein interacts with two other checkpoint control proteins, HHUS1and HRAD1. We also found that a region near the beginning of the protein controls programmed cell death (called apoptosis), a cell suicide response to DNA damage. Therefore, we developed a hypothesis about the function of the protein, based on our results. We believe that the protein has two functional domains, one involved in mediating cell death and the other enhancing cell survival after irradiation or chemical exposure by controlling cell cycle delays. Thus, this gene is an important determinant of the response of cells to radiation or chemicals and likely mediates the decision to induce apoptosis when damage is extensive or delay cell cycle progression and promote survival when damage is minimal and repairable. These results are part of the larger project wherein a detailed analysis of the structure of the gene and encoded protein have also been determined. Thus, we established a role for

HRAD9 in two major cellular pathways, apoptosis and checkpoint control, and developed as well as justified the foundation for assessing the status of this gene in individuals as a predictor of their genetic proness to adverse health effects caused by radiation or chemical exposure.

4. **Research Objectives:**

The project was conducted to establish *HRAD9* as a genetic marker capable of reflecting whether an individual would be genetically predisposed to deleterious health effects caused by radiation or chemical exposure. Analyses of the structure and function of *HRAD9*, as outlined in this project, provides the foundation to pursue the detection of mutations in this gene as a potential indicator of susceptiblity. Several human genes known to mediate resistance to radiations and chemicals in addition to *HRAD9* have been isolated (Friedberg et al., 1995; Yu et al., 1999). Furthermore, numerous studies have begun to evaluate the frequency of polymorphisms found in these genes in the general population, as well as their biological impact (Berwick and Vineis, 2000). The innovative aspects of this project lie especially in the function of this gene, making it a prime candidate for playing a significant role in determining susceptibility to DNA damage. *HRAD9* plays an important role in promoting resistance to chemical and physical agents, and uniquely regulates two major DNA damage response pathways, checkpoint control and apoptosis. Other organizations have supported the isolation and characterization of the fission yeast *rad9* gene, which served as the foundation for studies involving *HRAD9*.

5. **Methods and Results:**

DNA sequence of *HRAD9* determined

We isolated human *HRAD9* cDNA and genomic DNA sequences by a combination of searching computer data bases for sequences homologous to yeast *rad9*, obtaining a partial cDNA clone from I.M.A.G.E. consortium, then using the insert via in situ hybridization to probe human cDNA and genomic DNA libraries. Sequence analysis of the 2.1 kbp cDNA obtained

revealed a 1,176 bp open reading frame encoding a protein containing 391 amino acids. The encoded protein is 25% identical and 52% similar at the amino acid level to *S. pombe* rad9, and 27% identical and 54% similar to the *S. octosporus* protein we previously identified. The predicted rad9 proteins exhibit sequence similarity over their entire lengths, suggesting that the human cDNA is full length. The entire DNA sequence of the genomic clone, which includes all of the cDNA sequences, was also determined and reveals that the gene contains 10 exons spanning approximately 5.5 kbp.

Isolation of the mouse *Mrad9* gene

Mrad9 cDNA and genomic DNA versions from 129/Sv//Ev mice have been isolated by using in situ hybridization with the *HRAD9* cDNA as a probe to screen corresponding libraries (Hang et al., 1998). The complete DNA sequence of each has been determined. The gene was localized to approximately 10 kbp of the originally isolated 15 kbp fragment of genomic DNA. It contains 10 exons and 9 introns, like the human version of the gene. Figure 1 compares the encoded amino acid sequences in *Mrad9* with those found in the other versions of the protein. The *Mrad9* gene product is a 42,041 dalton protein containing 389 amino acids. The mouse protein is 82% identical to the human version and 88% similar at the amino acid level. The *Mrad9* encoded protein is 27% identical and 56% similar to the *S. pombe* protein. Several regions completely conserved throughout evolution are apparent, and are likely to be important for function. These sequences are being used for the construction of *Mrad9* knockout mice to serve as a model for establishing the role of the gene product in the response of mammals to radiation or chemical exposure.

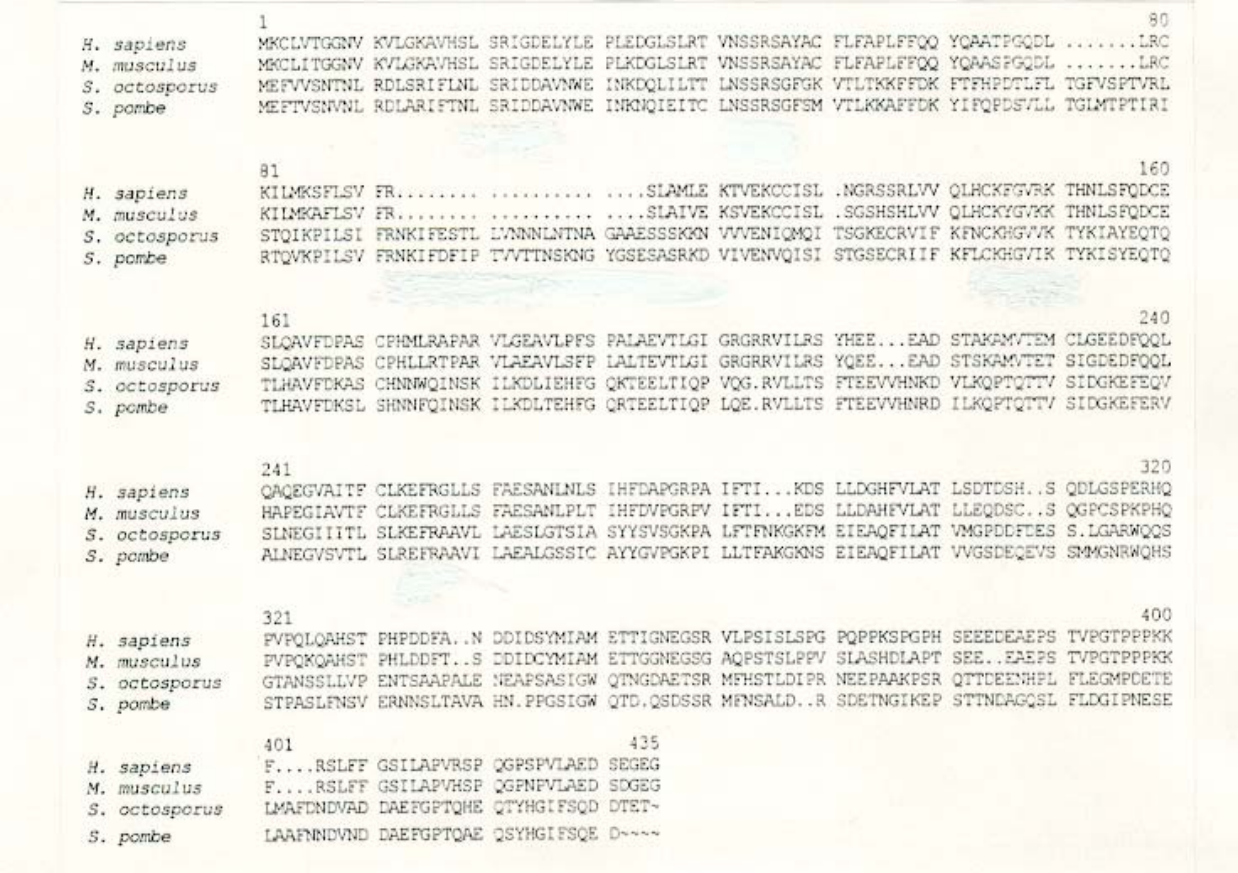

Figure 1. Comparison of amino acid sequences of rad9 proteins from different organisms. Lines between amino acids indicate identical molecules; dots indicate amino acids that are not identical but have similar physiochemical properties.

HRAD9 polymorphism detection in radiosensitive radiotherapy patients

A small subset of individuals undergoing radiotherapy (for example, to treat prostate cancer) exhibit extreme radiosensitivity demonstrated as severe late effects appearing several years after treatment. A study to examine the role of *HRAD9* in mediating this radiation sensitivity has begun, and has involved assessing the status of the gene in these patients and in suitable controls, using standard molecular techniques. RNA has been isolated from blood cells, RT-PCR with *HRAD9*-specific primers was performed, and amplified cDNAs were digested with restriction enzymes and fractionated on SSCP gels to identify fragments with mutations. The DNA sequence of *HRAD9* fragments bearing mutations, as indicated by SSCP, was also determined to

confirm the initial SSCP studies and to identify the nature of the alterations -i.e., whether they occur in the coding region of the gene and result in a dramatic alteration of the protein. Results of this preliminary study using only five such patients are illustrated in Fig. 2, and future studies will involve an analysis of larger populations.

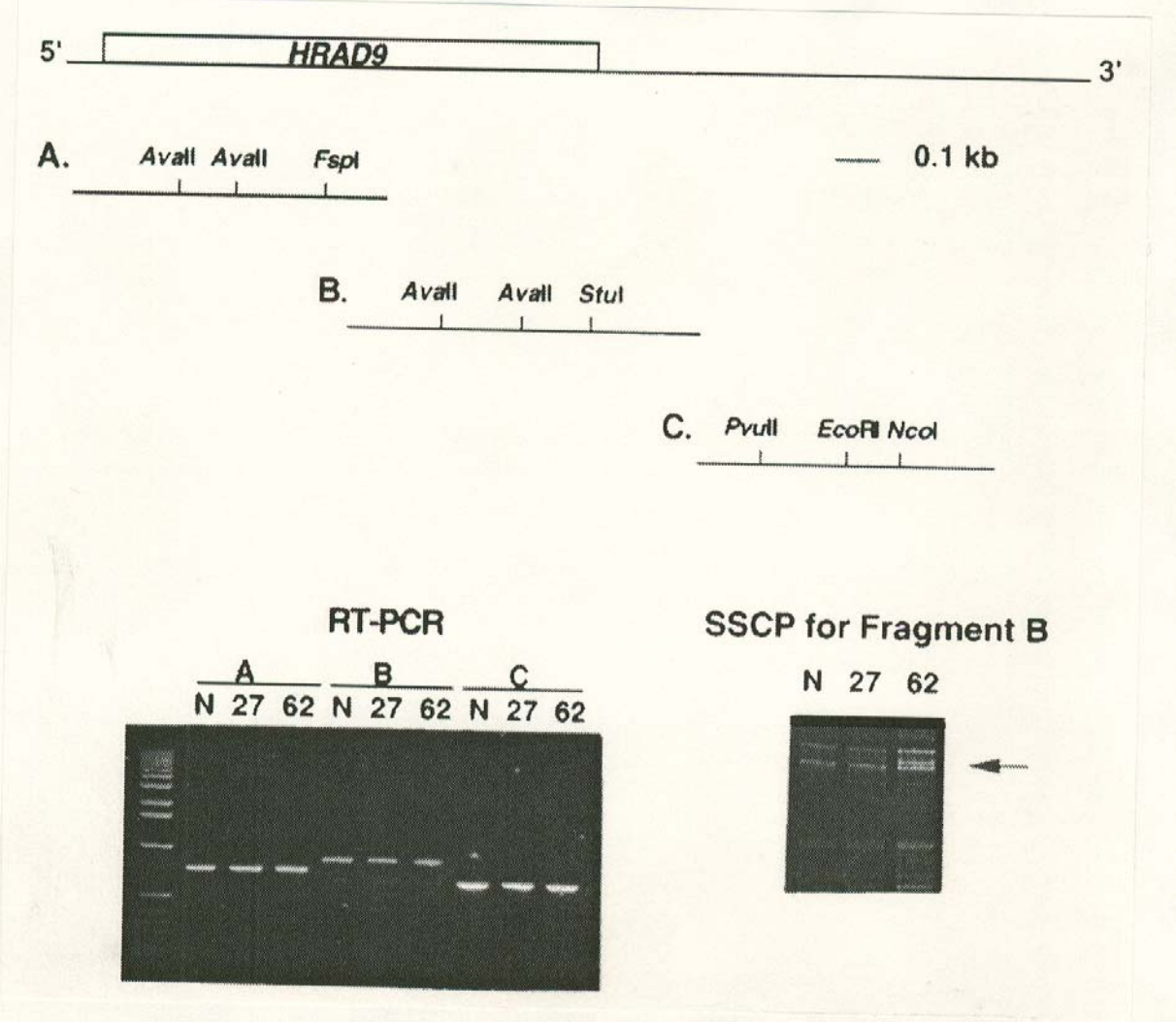

Figure 2. Detection of *HRAD9* polymorphisms by RT-PCR and SSCP analysis in cells from individuals that were treated for prostate cancer by radiotherapy and exhibited severe adverse late effects reflecting abnormal radiosensitivity. Top: *HRAD9* cDNA is amplified as three fragments (A, B and C) using RNA from cells as a template and suitable *HRAD9* oligonucleotide primers. Lower left: Amplification of *HRAD9* cDNA by RT-PCR as A, B, and C fragments

from normal (N) control or patient (numbers 27 and 62) cell samples. Lower right: SSCP analysis of the B fragments in the left panel. The arrow indicates the extra band in the B fragment of number 62, which reflects a polymorphism relative to normal or patient 27 *HRAD9*. Sequence analysis confirmed this alteration and indicated that a C to T transition occurred.

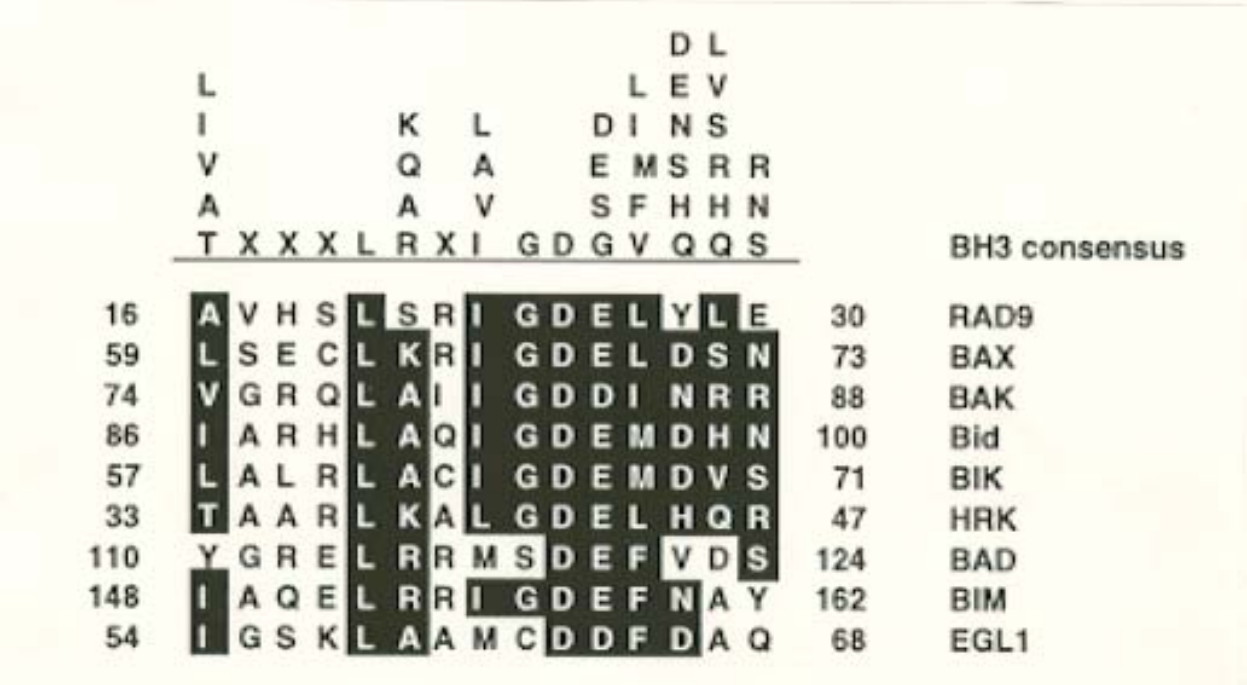

Figure 3. Alignment of amino-acid residues in the BH3-homology regions of Bcl-2-family members and human HRAD9. Comparison of the BH3-like regions in HRAD9 and Bcl-2-family members (human HRAD9, GenBank accession number U53174; human BAX, L22474; human BAK, U23765; mouse Bid, U75506; human BIK, U34584; human HRK, U76376; human BAD, AF021792; human BIM, AF032457; *Caenorhabditis elegans* EGL1, AF057309). Amino acids that match the BH3 consensus (accession number PS01259) are darkly shaded.

HRAD9 has pro-apoptotic function

In collaboration with Dr. Wang's group at the Moffitt Cancer Center in Florida, Dr. Lieberman's lab found that *HRAD9* has pro-apoptotic function (Komatsu et al., 2000). The HRAD9 protein contains a BH3-like domain, at amino acids 16-30 (Fig. 3), that is capable of

binding the anti-apoptotic proteins BCL-2 and BCL-xL. And, this domain is responsible for inducing apoptosis when *HRAD9* is overexpressed (Fig. 4). Overexpression of BCL-2 in parallel neutralizes this HRAD9-mediated pro-apoptotic activity, suggesting that the relative levels of these proteins can regulate apoptosis. Thus, we provide evidence that in addition to its classic checkpoint control function, *HRAD9* also possesses a novel apoptotic activity.

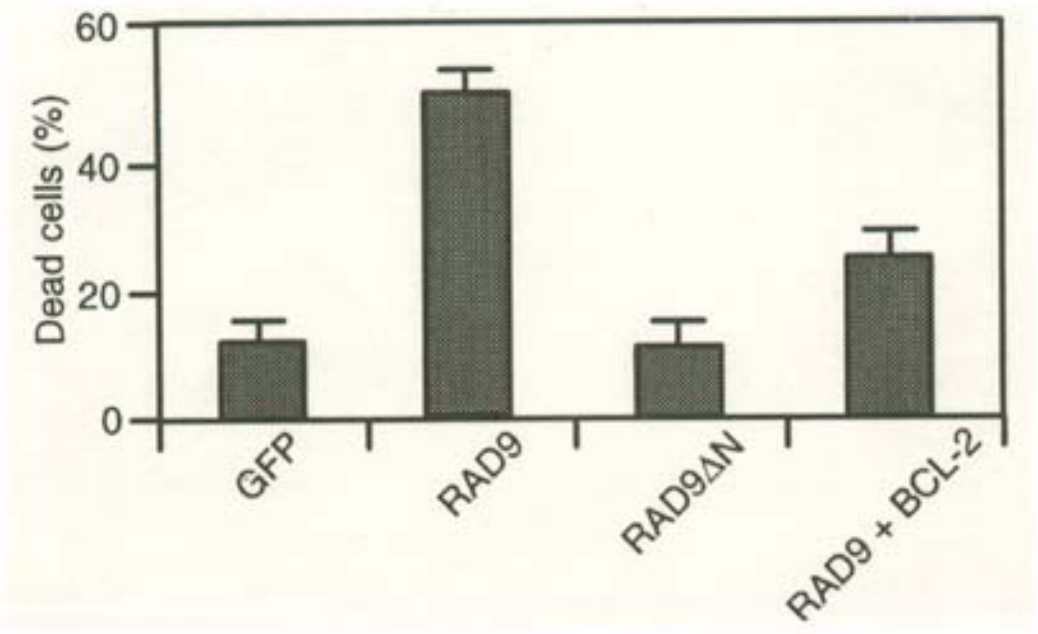

Figure 4. HRAD9 promotes apoptosis in a BH3-dependent manner. MCF7 cells were transfected with 1 mg plasmids encoding GFP–HRAD9, GFP–HRAD9DN or control GFP, together with 2 mg pRC/CMV–BCL-2 or parental plasmid DNA. 2 days after lipofectamine transfection, the percentage of GFP-positive dead cells was determined by propidium iodide staining (data shown are means ± s.d.; $n = 3$).

Physical interaction of HRAD9 with human checkpoint control proteins

Previous work by Kostrub et al. (1998) indicated that *S. pombe* Rad1 and Hus1 proteins interact in a *rad9*-dependent manner. Based on this finding, we and others (Volkmer and Karnitz, 1999; St. Onge et al., 1999; Hang and Lieberman, 2000) found that the human version of all three of these proteins physically interact. Furthermore, we found that they bind to each

other directly in all pairwise combinations, and not just as part of a multi-protein complex. We also defined regions of each protein important for those interactions. Of particular importance to the function of HRAD9 are the findings that the C-terminal portion of this checkpoint protein is essential for interacting with HHUS1p and the C-terminal region of HRAD1p. And, since the N-terminal portion of HRAD9 protein was previously demonstrated by us to regulate apoptosis (Komatsu et al., 2000), this protein likely has at least two functional domains, one that regulates programmed cell death and the other cell cycle checkpoint control. Therefore, these results support the hypothesis that HRAD9 coordinately regulates two major processes central to the response of cells to DNA damage.

In vitro mutagenesis of *S. pombe rad9* to identify protein regions important for function

S. pombe rad9 mutations can render cells sensitive to hydroxyurea, gamma-rays and UV light, and eliminate associated checkpoint controls. In vitro mutagenesis was performed on *S. pombe rad9*, and altered alleles were transplaced into the genome to ascertain the functional significance of five groups of evolutionarily conserved amino acids (Hang et al., 2000). Most targeted regions were changed to alanines, whereas *rad9*-S3 encodes a protein devoid of 22 amino acids normally present in yeast but absent from mammalian Rad9 proteins. We examined whether these *rad9* alleles confer radiation and hydroxyurea sensitivity, and whether the sensitivities correlate with checkpoint control deficiencies. One *rad9* mutant allele was fully active, whereas four others demonstrated partial loss of function. *rad9*-S1, which contains alterations in a BH3-like domain, conferred hydroxyurea resistance but increased sensitivity to gamma-rays and UV light, without affecting checkpoint controls. This is consistant with the function of this region in apoptosis. *rad9*-S2 reduced UV sensitivity marginally, without altering other phenotypes. Two alleles, *rad9*-S4 and *rad9*-S5, reduced hydroxyurea sensitivity, radiosensitivity and caused aberrant checkpoint function. Hydroxyurea-induced checkpoint control could not be uncoupled from drug resistance. These results establish unique as well as overlapping functional domains within Rad9p and provide evidence that requirements of the

protein for promoting resistance to radiation and hydroxyurea are not identical. Future work will make use of this information to analyze the corresponding conserved regions in *HRAD9*, and to provide the basis for determining whether specific mutations found in the gene have functional relevance.

6. **Relevance, Impact and Technology Transfer:**

a. A critical DOE environmental management problem involves reducing the health risks of workers who participate directly in the cleanup of hazardous wastes at DOE sites. The knowledge gained from the current study, about the structure of *HRAD9* and its dual function in controlling resistance to DNA damaging agents, forms the foundation to develop the use this gene as a biomarker for determining genetic susceptibility to hazardous wastes.

b. The scientific knowledge generated by this study will lead to an improved ability to identify individuals at exceptional risk for developing deleterious health effects caused by radiation or chemical exposure.

c. This new scientific knowledge impacts on broad, fundamental scientific research goals and is also timely in meeting the needs of DOE. It advances the understanding of molecular mechanisms that govern the response of individuals to DNA damage and simultaneously can be used pragmatically as a means to identify those individuals at high risk for developing adverse health effects in response to radiation or chemical exposure.

d. The impact of the results is that they can be used to develop a test to identify individuals susceptible to the deleterious health effects of radiation or chemical exposure, and thus limit the exposure of those individuals to hazardous agents. For example, the status of *HRAD9* can be examined in blood samples from individuals by standard molecular methodologies such as single-strand conformation polymorphism analyses, DNA sequencing, or denaturing high pressure liquid chromotography. Those persons found to contain mutated forms of the gene, especially thought to be effected in a functional region, would be counciled to avoid or reduce exposure to radiations or chemicals.

e. Larger scale trials are warranted to assess the prevalence of *HRAD9* alterations in the general population and their biological impact.

f. This research has lead to a collaborative efffort between the P.I. of this project, Dr. Lieberman, and the P.I. of a separate EMSP project, Dr. Richard Albertini. These individuals are each interested in studying biomarkers for genetic susceptibility to DNA damaging agents, and are currently planning a joint project that focuses on this common area of concern.

g. This research has lead to a better understanding of the molecular mechanisms that govern susceptibility to radiations and chemicals. In particular, it has provided evidence that *HRAD9* plays a major role in two important processes, chcckpoint control and apoptosis, which are central for determining the consequences of incurring DNA damage.

h. No other hurdles must be overcome. Testing of DOE worker blood samples for the status of *HRAD9* (as well as other radio/chemoresistance genes) can commence at any time.

i. No other government agencies or private enterprises have been contacted about the applicability of this project. However, Dr. David Thomassen of the DOE Low Dose Radiation Program is interested in broader, followup, population-based investigations.

7. **Project Productivity:**

Most of the aims of the original proposal were accomplished, especially the structural analyses of *HRAD9*. Results from this study indicating that the gene plays a role in apoptosis were unexpected and important. Therefore, much of our efforts in the latter part of the award period were devoted to more specifically characterizing the function of *HRAD9* in programmed cell death.

8. **Personnel Supported:**

Mr. Michael Chaplin, Dr. Charles Geard, Dr. Haiying Hang, Dr. Tom Hei, Mr. Kevin Hopkins, Dr. Howard Lieberman, Ms. Sarah Rauth, Mr. Wei Zheng

9. **Publications:**

Published in peer-reviewed journals

Hang, H., S. J. Rauth, K.M. Hopkins, S.K. Davey, and H.B. Lieberman. (1998) Molecular cloning and tissue-specific expression of *Mrad9*, a murine orthologue of the *Schizosaccharomyces pombe rad9*$^{+}$ checkpoint control gene. J. Cell. Physiol. 177:241-247.

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Komatsu, K., T. Miyashita, H. Hang, K.M. Hopkins, W. Zheng, S. Cuddeback, M. Yamada, H.B. Lieberman, and H.-G. Wang. (2000) Human homologue of *S. pombe* Rad9 interacts with Bcl-2/Bcl-x_L and promotes apoptosis. Nature Cell Biol. 2:1-6.

Hang, H., and H.B. Lieberman. (2000) Physical interactions among human checkpoint control proteins HUS1p, RAD1p and RAD9p, and implications for the regulation of cell cycle progression. Genomics 65:24-33.

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10. **Interactions:**

a. Results of this study were presented at several annual Radiation Research Society meetings, a DOE workshop, and in our Center's Annual Reports.

b. No consultative or advisory functions were a direct outgrowth of this project.

c. This work has lead to a collaborative effort with Dr. Richard Albertini at the University of Vermont to investigate and validate the use of *HRAD9* and other markers at the human population level for predicting susceptibility to the deleterious health effects potentially inducible by exposure to radaitions or chemicals.

11. **Transitions:**

Not yet applicable, although the goal is the use *HRAD9* and related genes as part of a genetic screen to identify individuals predisposed to the development of deleterious health effects after exposure to radiations or hazardous chemicals.

12. **Patents:**

None

13. **Future Work:**

The structure and function of *HRAD9* is well characterized as a result of this project. Although these types of studies will however continue, future work will focus primarily on bringing the investigation of *HRAD9* to the level of human populations. In particular, we are interested in examining the status of *HRAD9* (and other radio/chemoresistance genes) with regard to polymorphisms in human populations. These studies will concern an analysis of the functional impact of *HRAD9* mutations when found. For example, the role of *HRAD9* mutations in mediating genetic susceptibility to DNA damage, in particular in leading to cancer, will be ascertained.

14. **Literature Cited:**

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15. **Feedback:**

I particularly found workshops helpful in terms of gaining a broader perspective of the program and facilitating collaborations.

16. **Appendices:**

None

17. **Quantities/Packaging:**